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Phenotypic and Functional Analysis of Positive Selection in the γ/δ T Cell Lineage

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Summary

Recent evidence suggests that T cells expressing γ/δ antigen receptors (T cell receptor [TCR]) are subject to positive selection during development. We have shown that T cells expressing a class I major histocompatibility complex (MHC)-specific γ/δ TCR transgene (tg) are not positively selected in class I MHC-deficient, β_2-microglobulin (β_2m) gene knockout mice (tg^+ β_2m^-). In this report, we examine phenotypic and functional parameters of γ/δ positive selection in this transgenic model system. TCR-γ/δ tg^+ thymocytes of mature surface phenotype (heat stable $antigen^-$, $CD5^{hi}$) were found in β_2m^+ but not in β_2m^- mice. Moreover, subsets of tg^+ thymocytes with the phenotype of activated T cells (interleukin [IL]$2R^+$, $CD44^{hi}$, or Mel-14^{lo}) were also present only in the β_2m^+ mice. Cyclosporine A, which blocks positive selection of TCR-α/β T cells, also inhibited γ/δ tg^+ T cell development. These results support the idea that positive selection of TCR-γ/δ requires active TCR-mediated signal transduction. Whereas tg^+ β_2m^+ thymocytes produced IL-2 and proliferated when stimulated by alloantigen, TCR engagement of tg^+ β_2m^- thymocytes by antigen induced IL-2R expression but was uncoupled from the signal transduction pathway leading to IL-2 production and autocrine proliferation. Overall, these results demonstrate significant parallels between γ/δ and α/β lineage development, and suggest a general role for TCR signaling in thymic maturation.

The final stages of T cell differentiation in the thymus are mediated through interactions between the TCR and self-ligands consisting of both MHC and non-MHC antigens (1, 2). The TCR–self-antigen interactions that lead to clonal deletion or inactivation (anergy) are referred to as negative selection and serve to maintain self-tolerance. TCR–self-antigen interactions also are required for maturation of thymocytes into the mature phenotypes that are fully functional and capable of exiting from the thymus and establishing the T cell repertoire of the peripheral lymphoid system. Thus, the differentiation of $CD4^+CD8^+$ thymocytes into $CD4^+$ $CD8^-$ or $CD4^-CD8^+$ single-positive T cells requires an interaction of the TCR with appropriate self-class II or -class I MHC antigens, respectively, expressed on thymic epithelial cells. This process, termed positive selection, has been illustrated in a number of experimental models, including TCR transgenic mice as well as mice that fail to express class I or II MHC antigens as a result of gene disruptions through homologous recombination (3–10). However, the molecular basis of signal transduction during positive selection, as well as the manner in which distinct TCR signals can lead alternatively to negative or positive selection in the thymus, are poorly understood.

We have recently established a model system demonstrating a requirement for positive selection of thymocytes expressing a class I MHC-specific transgenic TCR-γ/δ (transgene [tg][1]). Tg^+ mice were bred to mice expressing no class I MHC because of the disruption of their β_2-microglobulin (β_2m) gene through homologous recombination. The resulting tg^+ β_2m^- offspring had tg^+ thymocytes that did not proliferate when stimulated through the TCR, and failed to exit from the thymus to populate peripheral lymphoid organs (11). Given that many non-MHC-specific γ/δ T cells

[1] *Abbreviations used in this paper:* β_2m, β_2-microglobulin; CsA, cyclosporine A; HSA, heat stable antigen; tg, transgene.

can mature normally in β_2m^- mice (12), these results were consistent with a maturational block in tg development due to the absence of a specific TCR–self-class I MHC molecule interaction, i.e., positive selection.

In an effort to understand the molecular basis of positive selection in the γ/δ lineage and to examine its relationship to α/β T cell differentiation, we have examined various phenotypic and functional characteristics of the tg^+ thymocytes from β_2m^+ versus β_2m^- mice. Flow cytometric analysis revealed notable differences in surface antigen expression between these related cell populations, which have allowed us to identify the changes in cell surface phenotype associated with γ/δ thymocyte maturation. Several markers of phenotypic maturation in the γ/δ lineage proved to be analogous to those denoting α/β thymocyte differentiation, suggesting that our model could provide insights of general relevance for positive selection of the TCR repertoire.

Materials and Methods

Animals. The γ/δ transgenic mice, as well as mice transgenic for a TCR-α/β specific for pigeon cytochrome c/I-E^k, have been previously described (4, 13), as have been the β_2m-deficient mice (7). BALB/c, C57Bl/6, and B10.BR mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or from the National Cancer Institute–Frederick Animal Research Facility (Frederick, MD).

Antibodies. FITC-conjugated antibodies used are as follows: anti-Vγ2, anti-α/β (both from Pharmingen, San Diego, CA), goat anti–hamster IgG and goat anti–rat IgG (both from Caltag Laboratories, San Francisco, CA). Biotinylated antibodies included anti-: heat stable antigen (HSA), Pgp-1 (CD44), CD45RB (16A), leukocyte cell adhesion molecule (LECAM) (Mel-14), IL-2R (all from Pharmingen), CD4 (Caltag Laboratories), and CD5 (Becton Dickinson & Co., Mountain View, CA). For fluorescent staining, hybridoma supernatants of anti-Vβ3 (KJ25) (14) and anti-Vα11 (RR8) (15) were used followed by the appropriate FITC-conjugated secondary antibody. Removal of $CD4^+$ and $CD8^+$ cells from thymic preparations used for staining and proliferation assays by magnetic bead separation was performed with hybridoma supernatants of GK1.5 and 53-6.72, respectively (American Type Culture Collection, Rockville, MD). Anti-CD28 used to stimulate proliferation was in the form of ascites (16, 17). Antibodies used for lymphocyte depletion of bone marrow cells used for chimera preparation are anti-Thy 1.2 (J1J) and anti-CD5 (Ly-1.2) (C3PO) (18) in the form of hybridoma supernatants. Anti-CD4 (RL172.4) and anti-CD8 (83-12-5) supernatants (the kind gift of L. A. Jones and A. Kruisbeek, Netherlands Cancer Institute) were used with complement to prepare thymocytes for the cyclosporine A (CsA) proliferation assay.

Cell Preparation. Thymuses were removed from each group of mice, pooled, and single cell suspensions made. Populations of $CD4^-CD8^-$ V$\gamma2^+$ thymocytes were purified by incubating thymic populations with rat IgG antibodies to CD4 (GK1.5) and CD8 (53-6.72), followed by incubation with magnetic beads coated with sheep anti–rat IgG (Dynabeads; Dynal, Inc., Great Neck, NY). Cell separation was performed by magnetic removal of $CD4^+$ and $CD8^+$ cells bound to the beads. Using this method, virtually 100% of the cells were $CD4^-CD8^-$ double negative and on average, 85% of the purified cell population was V$\gamma2^+$. BALB/c, CD57Bl/6, and B10.BR splenic cells used as APCs were processed into a single cell suspension, and RBCs were lysed with ACK lysis buffer (Biofluids Inc., Rockville, MD). Spleens were irradiated with 2,000 rad. Bone marrow cells for generation of chimeras were depleted of mature lymphocytes by complement-mediated lysis with anti-Thy1.2 and anti-Ly-1.2 antibodies plus complement.

Fluorescence Staining and FACS® Analysis. Staining was performed using standard techniques. Briefly, 0.5–10^6 cells were first incubated with FITC-conjugated antibodies or hybridoma supernatants followed by the appropriate FITC-conjugated anti-Ig. Cells were then incubated with biotinylated antibodies followed by streptavidin conjugated to PE or allophycocyanin (Cal-biochem-Novabiochem Corp., La Jolla, CA). In some experiments, cells were fixed with 1% paraformaldehyde after staining. Flow cytometric analysis was performed using either a FACS® (model 440; Becton Dickinson & Co.; see Figs. 4 and 5, experiment 1); an Epics (Coulter Electronics Inc., Hialeah, FL; see Figs. 1, 2, and 6); or a FACScan® (Becton Dickinson & Co.; see Fig. 5, experiment 2). FACS® analyses were performed either on selected double negative (DN) thymocytes or on whole thymocyte populations, depending on the total number of thymocytes available for analysis. However, the results obtained using either whole thymocyte populations or purified DN cells were always qualitatively equivalent.

Proliferation Assays. 5×10^4 $CD4^-CD8^-$ tg^+ thymocytes per well were plated in flat-bottomed microtiter plates in complete media. 5×10^5 irradiated spleen cells/well from either BALB/c or C57Bl/6 mice were used as APCs. Human recombinant IL-2 (Cetus Corp., Emeryville, CA) was added at a final concentration of 50 U/ml. 1 μM ionomycin (Calbiochem-Novabiochem) and/or 10^{-7} M PMA (Sigma Immunochemicals, St. Louis, MO) were added to 5×10^4 thymocytes without APCs. Anti-CD28 antibody was used as a 1:500 dilution of ascites. Assays were done in triplicate and cells were cultured for 48 h at which time 1 μCi of [^{3}H]thymidine was added. After further incubation for 12–16 h, proliferation was measured as incorporation of [^{3}H]thymidine in cpm.

CsA Treatment of Bone Marrow Chimeras. BALB/c or B10.BR mice were irradiated (850 rad) using a Cs source at day 0. The following day, 0.5–10^7 lymphocyte-depleted bone marrow cells from TCR-γ/δ (BALB/c) or TCR-α/β (B10.BR) transgenic mice were injected intravenously into the irradiated recipients. On day 3, daily intraperitoneal injections of CsA (20 mg/kg) or olive oil as a control were begun. After CsA treatment for the amount of time indicated in the figure legends, thymuses and spleens were removed and evaluated by flow cytometry.

Results

Phenotypic Changes Associated with Positive Selection of γ/δ tg^+ Thymocytes. Studies of TCR-α/β lineage development have shown that T cell maturation in the thymus is accompanied by progressive changes in the expression of a variety of cell surface proteins (19, 20). These observations have been useful in purifying distinct subsets of thymocytes and in examining their functional properties. The interactions of these cell surface molecules with their ligands may mediate signal transduction events important for differentiation or may determine appropriate cell migration, either within the thymus or to peripheral lymphoid organs.

We performed flow cytometric analysis to determine whether we could identify phenotypic differences between positively selected γ/δ tg^+ thymocytes from β_2m^+ mice and nonpositively selected tg^+ cells from β_2m^- mice. Analyses

were performed either on whole thymocyte populations or on selected $CD4^-CD8^-$ (DN) thymocytes. The expression patterns of each surface marker shown are representative of several independent experiments.

The HSA is a heavily glycosylated cell surface protein that is expressed at high levels on immature thymocytes and is progressively downregulated during thymic maturation, such that the most mature thymocytes and peripheral T cells are HSA^- (21). Recent studies have shown that HSA serves as a maturational marker for γ/δ as well as for α/β T cells (12). For example, we (unpublished observations) and others (22) have found that only HSA^- γ/δ thymocytes proliferate in response to TCR stimulation. Therefore, we examined tg^+ thymocytes from β_2m^+ and β_2m^- mice for HSA expression. The tg^+ thymocytes from β_2m^+ mice consisted of both HSA^+ and HSA^- subsets (Fig. 1). Although the percentage of tg^+ HSA^- thymocytes was variable among individual β_2m^+ mice, a significant number of tg^+ HSA^- cells was consistently observed. In contrast, the tg^+ β_2m^- thymocyte population was reproducibly almost entirely HSA^+ (Fig. 1), consistent with a maturational block resulting from the absence of positive selection. Several other surface markers associated with thymic development were also differentially expressed by tg^+ β_2m^+ versus β_2m^- thymocytes. The level of CD5 expression also distinguishes immature from mature α/β thymocytes, rising progressively during α/β thymic maturation (23). Accordingly, we examined CD5 expression on the γ/δ thymocytes, and found that it was expressed at higher levels on tg^+ β_2m^+ than on tg^+ β_2m^- cells. Mean fluorescence intensity of CD5 was 33.8 on β_2m^+ thymocytes compared with 28.4 in β_2m^- mice (25% greater expression on β_2m^+ thymocytes). It is interesting that unlike α/β T cells, CD5 expression is dull on peripheral tg^+ γ/δ T cells (data not shown). Also, a significant subset of tg^+ β_2m^+, but not tg^+ β_2m^- thymocytes expressed the CD45RB antigen, which has recently been shown to be expressed on a subset of late $CD4^+CD8^+$ thymocytes expressing intermediate levels of α/β TCR (24). Using TCR-α/β transgenic models, intrathymic CD45RB expression was also recently shown to correlate with positive selection in the α/β lineage (25).

We also found differential expression of cell surface markers associated with T cell activation between the tg^+ β_2m^+ and β_2m^- thymocyte populations (Fig. 2). Both IL-2 receptors and CD44, which are upregulated after T cell activation (26, 27), were expressed at high levels on distinct subpopulations of tg^+ β_2m^+ cells. Three distinct levels of CD44 expression (high, intermediate, and low) were observed (Fig. 2). Tg^+ cells expressing the highest level of CD44 ($CD44^{hi}$), corre-

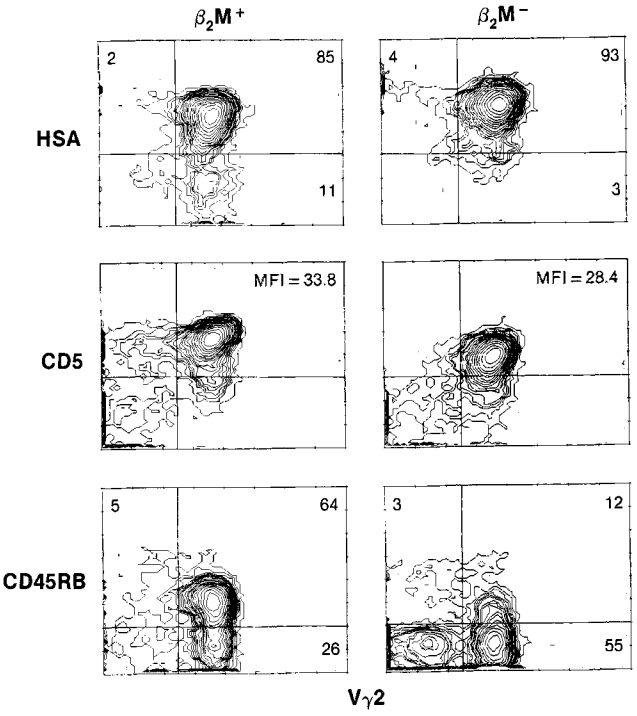

Figure 1. Surface phenotype analysis of differentiation antigens on transgene-bearing thymocytes in β_2m^+ and β_2m^- mice. $CD4^-CD8^-$ thymocytes purified by magnetic bead separation (see Materials and Methods) were analyzed by two-color flow cytometry for expression of maturation markers on tg^+ cells. The FACS® profiles of the various surface markers shown are taken from different experiments, but β_2m^+ and β_2m^- profiles are from the same experiment for each parameter. The results shown for each surface marker were verified in several experiments and were highly reproducible. (*MFI*) mean fluorescence intensity.

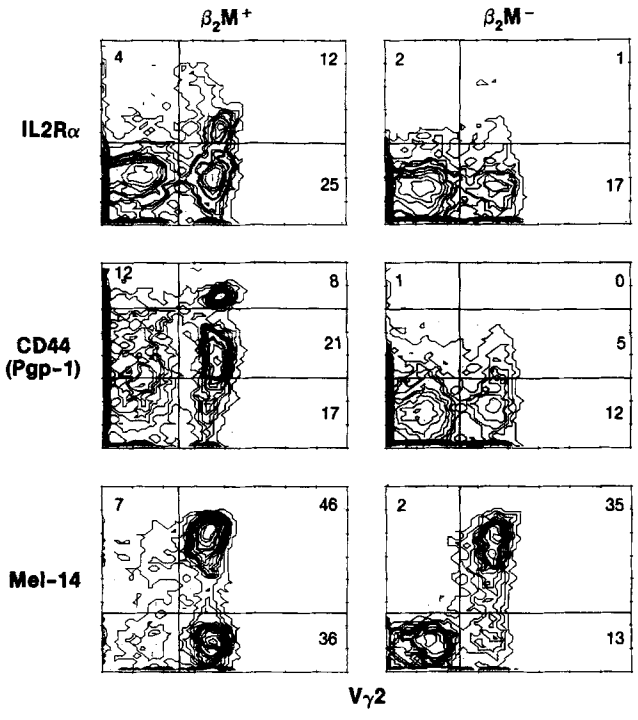

Figure 2. Subsets of tg^+ thymocytes from β_2m^+ but not β_2m^- mice express a surface phenotype characteristic of cell activation. Two-color flow cytometry as described in Fig. 1 was used to compare the surface expression of CD44, IL2R, and Mel-14 on $V\gamma2^+$ thymocytes from each group of mice. Evaluation of IL-2Rα and CD44 expression was performed on whole thymus. Mel-14 expression was examined on $CD4^-CD8^-$ thymocytes purified by magnetic bead separation.

sponding to the level of CD44 expression seen after activation of mature T cells (26) (see Fig. 6) constituted 8% of total thymocytes in the β_2m^+ mice in the experiment shown (Fig. 2). The IL-2R$^+$ and CD44^{hi} populations were found to be distinct by virtue of the fact that all the IL-2R$^+$ cells were HSA$^+$, whereas the CD44^{hi} cells were found predominantly among the more mature HSA$^-$ thymocytes (data not shown). In addition, a subset of tg$^+$ β_2m^+ thymocytes were Mel-14^{lo}. Mel-14, the lymph node–specific L-selectin homing receptor, is also transiently downregulated after T cell activation (28, 29). In contrast with the expression pattern of these cell surface molecules on tg$^+$ β_2m^+ thymocytes, there were few tg$^+$ β_2m^- thymocytes that were either IL-2R$^+$, CD44^{hi}, or Mel-14^{lo} (Fig. 2). However, a variable percentage of tg$^+$ β_2m^- thymocytes were CD44^{int} (see Fig. 6). The fact that differential IL-2R expression between tg$^+$ β_2m^+ and tg$^+$ β_2m^- cells is already observed within the HSA$^+$ population suggests that the TCR interactions leading to positive selection of γ/δ cells may begin at a relatively early stage of differentiation. Moreover, the activated phenotype observed during thymic selection is transient, as tg$^+$ cells in peripheral lymphoid organs are IL-2R$^-$, CD44^{lo}, and Mel-14^{hi} (data not shown).

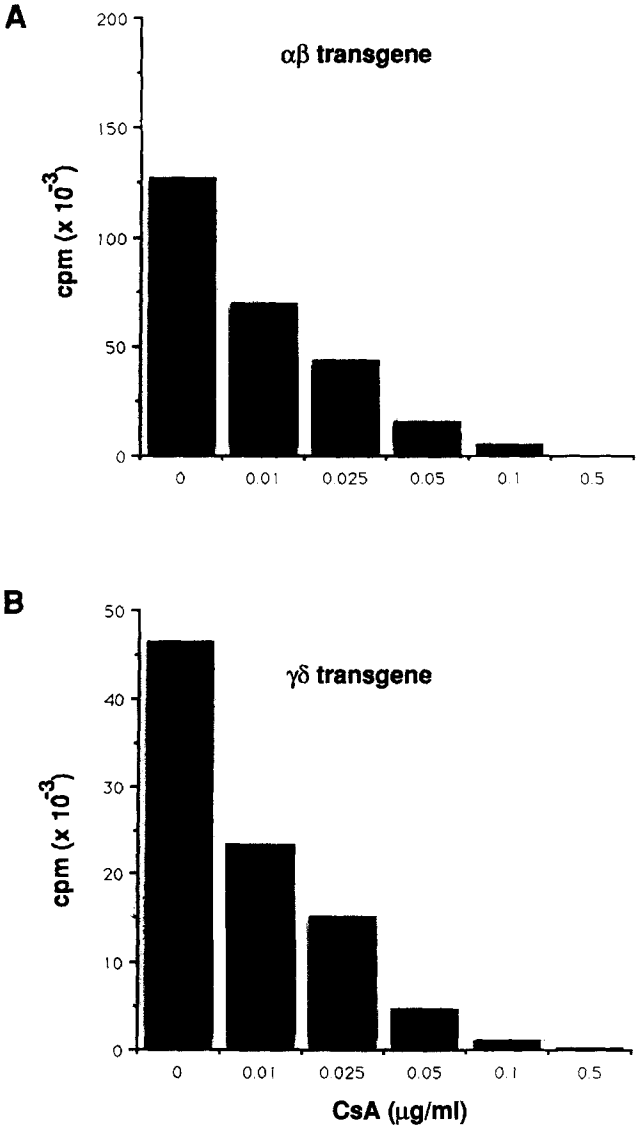

Figure 3. Inhibition of antigen induced proliferation of α/β and γ/δ transgenic thymocytes by CsA. Purified tg$^+$ thymocytes were cultured in the presence of antigen and various concentrations of CsA. Proliferation was measured as incorporation of [^{3}H]thymidine as described in Materials and Methods. (*A*) 10^5 purified cytochrome c–specific CD4$^+$ α/β tg$^+$ thymocytes (obtained by incubation with anti-CD8 antibody and complement) were cultured with 5 × 10^5 syngeneic H-2^k B10.BR APCs, 2.5 μM cytochrome c, and various concentrations of CsA. Proliferation of tg$^+$ cells to syngeneic APC alone was 335 cpm. (*B*) 2.5 × 10^4 γ/δ tg$^+$ thymocytes were cultured with 5 × 10^5 allogeneic H-2^b APCs and various concentrations of CsA. cpm with syngeneic H-2^d BALB/c APCs were 159.

γ/δ Thymocyte Development Is Influenced by CsA. Although the signals that mediate positive selection of TCR-α/β thymocytes are ill-defined, it is known that the process is sensitive to the inhibitory effects of CsA. For example, experiments in which CsA was administered to mice after irradiation and bone marrow reconstitution have shown that α/β thymocyte development is blocked at the CD4$^+$CD8$^+$ stage, before the selection of mature single-positive thymocytes (30–32). In contrast, these studies showed that the appearance of TCR-γ/δ–bearing CD4$^-$CD8$^-$ thymocytes was apparently unaffected by CsA, and therefore it was suggested that γ/δ development might not require positive selection.

However, in view of our evidence for positive selection in the γ/δ lineage, we decided to investigate further the potential effect of CsA on γ/δ development. Initially, we examined whether the antigen-induced proliferation of γ/δ tg$^+$ thymocytes was inhibited by CsA. The proliferative response of purified γ/δ tg$^+$ CD4$^-$CD8$^-$ thymocytes to antigen (H-2^b APCs) was assessed in the presence of various concentrations of CsA (Fig. 3) in direct comparison with the inhibition by CsA of the antigen-induced proliferation response of CD4$^+$ α/β tg$^+$ thymocytes specific for pigeon cytochrome c/I-E^k (4). As shown, the antigen-specific proliferation of both γ/δ and α/β transgenic thymocytes was inhibited at identical concentrations of CsA (Fig. 3). Thus, signal transduction through the γ/δ tg is sensitive to CsA.

To readdress the issue of CsA and γ/δ T cell differentiation, we reconstituted irradiated BALB/c mice with lymphocyte-depleted γ/δ tg$^+$ bone marrow, and examined thymocyte development in CsA-treated and control animals, according to previous protocols (31, 32). Flow cytometric analysis was performed on thymocyte populations 3–4 wk after bone marrow reconstitution. CD4 or CD8 single-positive TCR-α/β thymocytes, observed in the control mice, were absent in the animals that had been treated with daily intraperitoneal injections of CsA (Fig. 4 *A*). In contrast, there were abundant TCR-γ/δ tg$^+$ CD4$^-$CD8$^-$ cells, expressing normal levels of TCR, in both CsA-treated and control animals. To rule out the possibility that this result could be attributed simply to the inability of CsA to inhibit maturation of tg-bearing thymocytes, we reconstituted irradiated B10.BR animals with bone marrow from mice expressing a class II

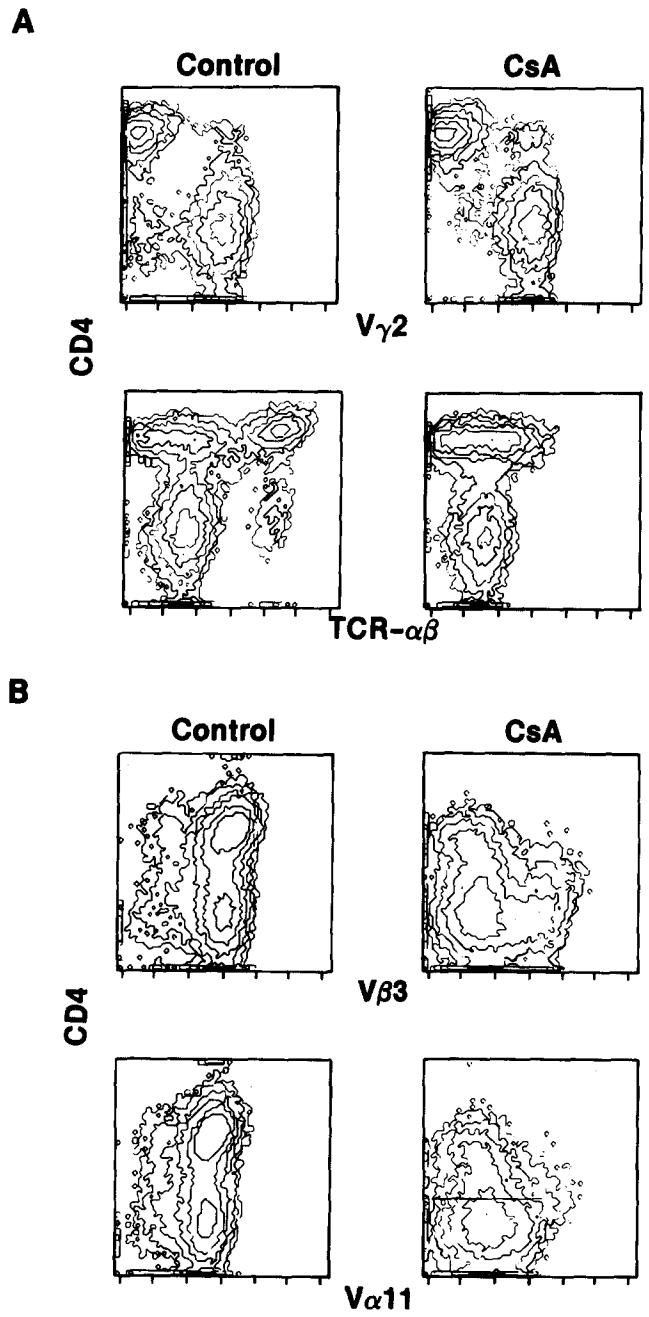

Figure 4. Effect of CsA on development of γ/δ and α/β transgene-bearing cells. Irradiated mice were reconstituted with lymphocyte-depleted bone marrow from γ/δ and α/β transgenic mice and then treated with CsA (20 mg/kg/d) or olive oil for 21 d. Thymocytes from these mice were then removed and examined for expression of the transgenic TCRs. (*A*) Lethally irradiated BALB/c (H-2^d) mice were reconstituted with bone marrow cells from mice expressing the γ/δ tg. After 21 d of intraperitoneal treatment with CsA or olive oil alone, thymocytes were removed and double-stained with anti-Vγ2 or anti-TCR-α/β and anti-CD4 mAbs. (*B*) Bone marrow cells from mice expressing a class II MHC (I-E^k) restricted pigeon cytochrome c-specific TCR-α/β tg were used to reconstitute lethally irradiated B10.BR mice. They then received daily intraperitoneal CsA or olive oil alone as above. Thymocytes were examined by two-color flow cytometry for expression of Vα11 or Vβ3 and CD4.

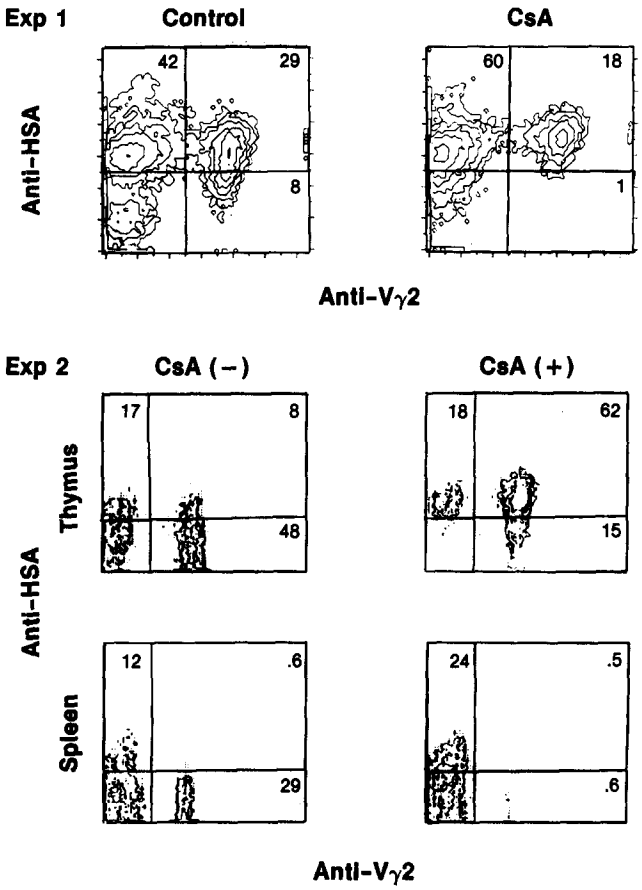

Figure 5. Effect of CsA on cell surface phenotype of TCR-γ/δ tg^+ thymocytes and on expression of the tg in peripheral lymphoid organs (*spleen*). Bone marrow reconstitution experiments were conducted as described in Fig. 4 and Materials and Methods using a CsA dose of 20 mg/kg/d. Thymocytes and spleen cells were evaluated after 4 wk (*Exp. 1*) or 8 wk (*Exp. 2*) by two-color flow cytometry for the surface expression of Vγ2 and HSA molecules.

MHC-restricted pigeon cytochrome c-specific TCR-α/β tg and examined the effect of CsA treatment on the development of α/β tg-bearing thymocytes (Fig. 4 *B*). As observed for nontransgenic TCR-α/β T cells (Fig. 4 *A*) (30–32), CsA treatment completely blocked the differentiation of α/β tg^+ thymocytes to the mature $CD4^+CD8^-$ phenotype. Thus, we conclude that CsA specifically does not inhibit the intrathymic appearance of $CD4^-CD8^-$ cells expressing normal levels of the TCR-γ/δ tg.

However, further analysis did show evidence of an inhibitory effect of CsA on γ/δ development (Fig. 5). Whereas a significant proportion of the tg^+ cells from the control thymuses of bone marrow-reconstituted mice were HSA^-, almost all the tg^+ thymocytes from CsA-treated litter mates were HSA^+ (Fig. 5). Thus, the percentage of γ/δ HSA^- tg^+ thymocytes was markedly reduced in mice treated with CsA after bone marrow reconstitution as compared with controls. It is interesting that a higher percentage of tg^+ HSA^- thymocytes was observed in the thymuses of bone marrow-transplanted mice examined 8 wk after reconstitution (Fig.

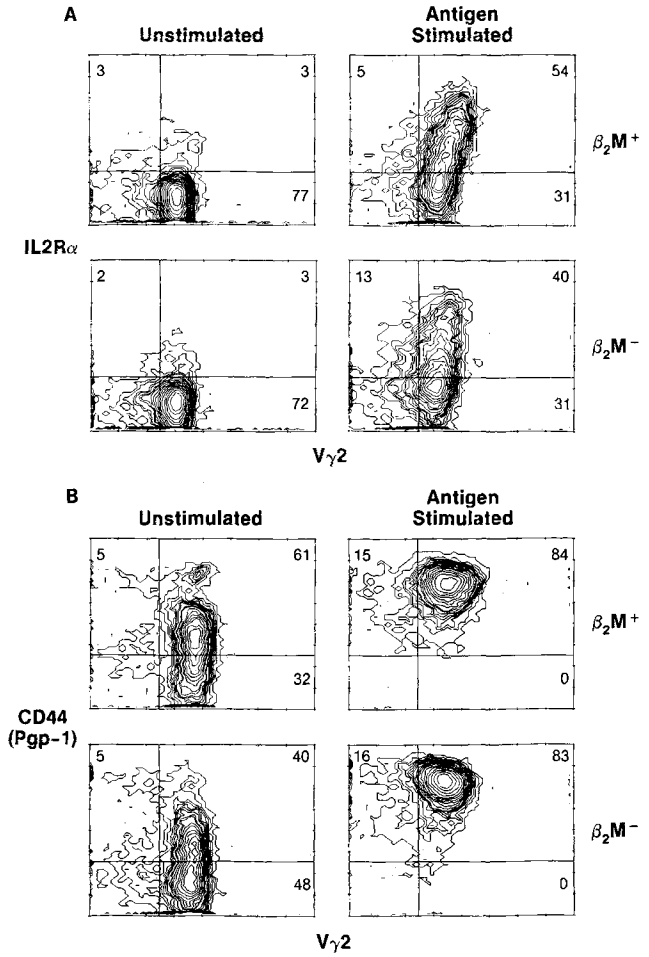

Figure 6. Tg-bearing thymocytes from both β_2m^+ and β_2m^- mice increase surface expression of IL-2R and CD44 after exposure to antigen. $CD4^-CD8^-$ tg^+ thymocytes purified by magnetic bead separation from β_2m^+ or β_2m^- mice were stimulated for 48–72 h with antigen ($H\text{-}2^b$ splenic APCs) and then evaluated by two-color flow cytometry for the expression of IL-2R or CD44 on $V\gamma2^+$ cells. Unstimulated cells were examined by flow cytometry either after incubation for 48–72 h with syngeneic ($H\text{-}2^d$) APCs (*IL2R*); or before any culture (*CD44*). In the experiment evaluating CD44 upregulation, tg-bearing cells were cultured in the presence of rIL-2 (50 U/ml) in addition to antigen. No upregulation of CD44 was observed with IL-2 alone (*data not shown*).

5, exp. 2) as compared with 4 wk after reconstitution (Fig. 5, exp. 1). We have consistently observed that the proportion of HSA^- tg^+ thymocytes increases with time after irradiation and bone marrow reconstitution. Although the reason for this is unclear, the observation is highly reproducible (Tatsumi, Y., J. Pena, L. Matis, D. DeLuca, and J. Bluestone, manuscript submitted for publication). Also, CsA treatment markedly reduced the number of tg-bearing cells in the peripheral lymphoid organs (Fig. 5, exp. 2). Overall, these results suggest that the differentiation induced by TCR-γ/δ interactions with a β_2m-associated self-antigen may indeed be inhibited by CsA, and therefore that, as for α/β development, positive selection of at least some γ/δ T cells is CsA sensitive.

Uncoupling of TCR-mediated Signals in γ/δ tg^+ Thymocytes from β_2m^- Mice. To explore the functional defect in the tg^+ β_2m^- thymocytes, we examined purified populations of tg-bearing DN cells activated by various specific and nonspecific stimuli. First, we examined surface phenotypic changes induced by antigen stimulation. We found that although, as previously reported (11), the tg^+ β_2m^- thymocytes do not proliferate when stimulated by antigen, the antigen stimulation of these cells did induce upregulation of IL-2Rα and CD44 (Fig. 6). Therefore, some components of TCR-mediated signal transduction are intact in these cells. It should be noted that the IL-2R expression observed on freshly isolated tg^+ thymocytes is absent after 2 d of in vitro culture in the absence of stimulation (Fig. 6). However, we do not believe that the IL-2R expression seen on the thymocytes stimulated with antigen ($H\text{-}2^b$ APCs) simply reflects a lack of IL-2R downregulation. First, IL-2R was also upregulated after antigen stimulation of the tg^+ β_2m^- thymocytes (Fig. 6), a population which initially does not express any IL-2R (Fig. 2). Second, we have evidence that IL-2R is upregulated after antigen stimulation of purified HSA^- tg^+ thymocytes, cells that do not express IL-2Rα (data not shown).

We next assessed the ability of the tg-bearing populations to proliferate in response to mitogenic stimuli. In concert with the ability of the tg^+ β_2m^- cells to upregulate IL-2R after antigen stimulation, the addition of exogenous IL-2 to antigen-stimulated cultures induced a strong proliferative response (Table 1). Thus, the IL-2–IL-2R signaling pathway is intact in these cells. A subpopulation of thymocytes present in β_2m^+ mice proliferated in response to exogenous IL-2 alone (Table 1). We presume but have not shown directly that these represent the tg^+ HSA^+ $IL\text{-}2R^+$ cells (Fig. 2). Few $IL\text{-}2R^+$ cells are present in tg^+ β_2m^- thymuses and, accordingly, they manifested little proliferative response to IL-2.

The significance of the HSA^+ $IL\text{-}2R^+$ phenotype in γ/δ tg development has not been established, and no clearly apparent homologous population has been identified in the α/β lineage, where the role of IL-2–IL-2R interactions during thymopoiesis remains controversial (33, 34). In normal thymuses, IL-2Rα is transiently expressed on a small popula-

Table 1. *Effect of IL-2 on Proliferation of γ/δ tg^+ Thymocytes*

	No IL-2		IL-2	
	β_2m^-	β_2m^+	β_2m^-	β_2m^+
	cpm			
APCs				
$H\text{-}2^d$	286	76	3,356	25,287
$H\text{-}2^b$	790	85,705	40,138	145,512

Response of tg^+ thymocytes of β_2m^+ and β_2m^- mice to exogenous IL-2. 5×10^4 $CD4^-CD8^-$ thymocytes from β_2m^+ or β_2m^- γ/δ tg^+ mice were incubated with syngeneic ($H\text{-}2^d$) or allogeneic ($H\text{-}2^b$) APCs. Recombinant human IL-2 was added at a concentration of 50 U/ml. Results are reported in uptake of ^3H in cpm. This experiment is representative of seven different experiments.

tion of HSA$^+$CD44$^-$CD4$^-$CD8$^-$TCR$^-$ cells with a significant capacity for thymus (e.g., all T cell lineages) reconstitution (35). Normally, IL-2R cannot be detected on more mature TCR$^+$ thymocytes (35, 36). However, IL-2R expression has also previously been observed at low levels on TCR-α/β tg$^+$ thymocytes (37). Thus, it cannot be ruled out that IL-2R expression on TCR$^+$ thymocytes will be a phenomenon associated exclusively with transgenic mouse models, perhaps related to the accelerated TCR expression during thymic differentiation seen in these animals. However, an intriguing model system was recently described in which an immature CD4$^+$CD8$^+$ TCR-α/β tg–expressing T cell line could be induced to differentiate in vitro to a CD4$^+$CD8$^-$ phenotype in the presence of cognate antigen and MHC (38). Among the phenotypic changes accompanying the apparent in vitro positive selection of this cell line were the upregulation of CD69, CD44, and CD25 (IL-2Rα). Thus, it remains of interest to determine whether IL-2 plays a critical role in T cell maturation.

To bypass the requirement for TCR-mediated signaling, tg$^+$ populations were activated with ionomycin and PMA. Under these conditions, the tg$^+$ β_2m$^-$ thymocytes showed significant, but reduced proliferation in comparison with their β_2m$^+$ counterparts (Table 2). We also studied signaling through the T cell costimulatory molecule CD28 (39–43). The expression of CD28 on developing thymocytes, the presence of the CD28 ligand B7 on thymic stromal cells, and the ability of thymocytes to be activated through the CD28 signaling pathway, all have implicated CD28 as having a role in thymic development. Using a murine CD28-specific mAb (16, 17) we determined that CD28 was expressed on both tg$^+$ β_2m$^+$ and β_2m$^-$ thymocytes (data not shown). Furthermore, stimulation with the CD28-specific mAb was synergistic with ionomycin and PMA in augmenting the proliferation of both populations (Table 2). This result indicated that the CD28 signaling pathway was functional in the tg$^+$ β_2m$^-$ thymocytes. In contrast with the effect of CD28 on ionomycin plus PMA activation, costimulation with CD28 mAb enhanced the antigen-induced proliferative response only in the tg$^+$ β_2m$^+$ thymic population (Table 2). No increase over background was observed when tg$^+$ β_2m$^-$ thymocytes were activated by antigen plus CD28-specific mAb. Finally, a previously reported mitogenic pathway induced by costimulation with CD28 and PMA (41) was also found to be functional in both the tg$^+$ β_2m$^+$ and β_2m$^-$ thymocytes (Table 2). However, whereas PMA plus CD28 together were strongly synergistic with antigen-induced proliferation in the β_2m$^+$ population, the addition of antigen produced no augmentation of the CD28 plus PMA-induced proliferation of tg$^+$ β_2m$^-$ thymocytes.

Thus, γ/δ tg$^+$ thymocytes that have not undergone positive selection have a profound defect in coupling TCR-mediated signals to autocrine proliferation pathways. However, TCR signaling leading to upregulation of the IL-2R is intact, as is the costimulatory pathway transduced through the CD28 molecule and the ability to respond to exogenous IL-2 after IL-2R upregulation.

Table 2. *Activation of γ/δ tg$^+$ Thymocytes*

Stimulation	Tg$^+$$\beta_2m^+$	Tg$^+$$\beta_2m^-$
Ionomycin	77	498
PMA	103	407
Ionomycin plus PMA	26,041	6,462
Ionomycin plus PMA plus CD28	45,724	18,195
Antigen (H-2^b APCs)	32,262	1,404
Antigen plus CD28	83,120	1,515
PMA plus CD28	23,713	19,948
Antigen plus PMA plus CD28	254,985	17,885
Antigen plus PMA	15,135	734

Proliferative response of tg$^+$ thymocytes from β_2m$^-$ and β_2m$^+$ mice to various stimuli. Assays were performed as described in Table 1 and in Materials and Methods to evaluate the response of CD4$^-$CD8$^-$ tg$^+$ thymocytes from each type of mouse to mitogenic stimuli. Experiments using PMA plus CD28 as stimuli were done in the presence of syngeneic (H-2^d) APCs.

Discussion

We have determined phenotypic and functional parameters that characterize the positive selection of thymocytes expressing a class I MHC–specific TCR-γ/δ transgene. Our data illustrate that the TCR–self-antigen interactions that occur during γ/δ positive selection induce demonstrable alterations in cell surface phenotype, and are required for the development of functionally mature IL-2–producing TCR-γ/δ T cells.

There are significant differences between α/β and γ/δ differentiation. For example, signaling through the CD4 and CD8 molecules, which plays a critical role in α/β thymocyte selection (7–10, 44), appears to have no role in γ/δ development. However, the results presented here provide evidence for at least some common mechanisms underlying both α/β and γ/δ differentiation. Thus, some of the phenotypic changes that accompany thymocyte maturation are shared by cells of the α/β and γ/δ lineages. It is notable that, as is the case for α/β T cells, the HSA molecule serves as a marker for γ/δ intrathymic development, such that only the HSA$^-$ TCR-γ/δ thymocytes are functionally mature and can exit from the thymus to populate peripheral lymphoid tissue (12). Accordingly, in the γ/δ tg$^+$ β_2m$^-$ mice, differentiation of tg-bearing thymocytes is arrested at the HSA$^+$ stage. In this light, we have also found that the recombination activating genes RAG-1 and RAG-2 are expressed in the HSA$^+$ TCR-γ/δ tg$^+$ thymocytes, but not in HSA$^-$ γ/δ tg$^+$ thymocytes (Tatsumi, Y. et al., manuscript submitted for publication), consistent with recent studies showing that RAG gene downregulation correlates with positive selection during α/β development (45, 46). Thus, downregulation of RAG gene expression may result from TCR–self-antigen interactions in both the α/β and γ/δ cell lineages. Furthermore, the patterns of expression of other cell surface molecules such as CD5, CD44, CD45, and Mel-14 also appear to have similarities

during thymic differentiation of γ/δ and α/β cells. Finally, we have shown that CsA can inhibit the development of the γ/δ as well as the α/β lineage.

The nature of the TCR-mediated signal transduction pathways induced during positive selection remains to be determined, but indirect evidence suggests that they may have some similarities to the signal transduction involved in mature T cell activation. Thus, both mature T cell activation and positive selection are inhibited by CsA (30–32, 47). Recent insights into the mechanism of immunosuppression by CsA have shown that the CsA–cyclophilin complex binds and inhibits the Ca^+-dependent phosphatase calcineurin (48–50) implying a critical role for this enzyme in the signal transduction pathway for IL-2 production. It will be interesting to determine whether this pathway is induced during thymic selection. Also, the *lck* kinase, whose function is critical for signal transduction in mature T lymphocytes (51), appears to have a central role in mediating α/β T cell differentiation (52). Further studies will examine if lck kinase activity is important for γ/δ T cell development.

It is interesting that several of the surface phenotypic changes associated with positive selection in our system (e.g., expression of IL-2R and CD44, downregulation of Mel-14) are characteristic of changes in phenotype that accompany activation of mature T cells (25–29). Similar observations have been made by Bendelac and co-workers (53, 54) in an examination of the activation events occurring during TCR-α/β thymic maturation. For example, these investigators identified a subpopulation of α/β lineage thymocytes at a late maturational stage (HSA^-) that expressed high levels of CD44. Based on the expression of other surface markers of activation, as well as the patterns of lymphokine secretion by discrete thymocyte subsets, Bendelac et al. (54) concluded that positive selection represents a transient but reversible TCR-mediated activation process that provides critical signals required for maturation.

The functional significance of all the phenotypic changes that accompany T cell development are presently unknown. Because several of the surface markers (CD45, HSA, and CD44) have been shown to have costimulatory properties (55–59), it is possible that in development they transduce signals important for differentiation. In addition, the homing receptor functions of molecules such as the L-selectins (Mel-14) and CD44 suggest a possible role in mediating intrathymic or extrathymic migration.

The signal transduction defect in the tg^+ β_2m^- thymocytes is characterized by an uncoupling of TCR signaling from the pathway leading to IL-2 production. This is a rather selective defect in that these cells can upregulate IL-2R when engaged by antigen and then proliferate in response to exogenous IL-2. They can also proliferate when TCR signaling is bypassed with ionomycin and PMA, although an additional defect is implied by the reduced magnitude of this proliferative response (Table 2). The functional properties of the tg^+ β_2m^- thymocytes are most closely related to those of a recently identified subset of murine HSA^+ TCR α/β single-positive thymocytes (53, 54), rather than the more immature $CD4^+CD8^+$ thymocytes that predominate when α/β positive selection is blocked. Murine $CD4^+CD8^+$ thymocytes activated through their TCR do not upregulate IL-2R nor respond to exogenous IL-2, although they do flux calcium (60–64). This could represent a difference in the molecular pathways governing positive selection of α/β versus γ/δ T cells. However, it has also been shown that human $CD4^+CD8^+$ thymocytes, like our tg^+ β_2m^- HSA^+ γ/δ thymocytes, can upregulate IL-2R and proliferate to IL-2 after TCR engagement (42). In any event, the selective defect in TCR signal transduction resulting from the blockade in γ/δ tg development in the β_2m^- environment should provide a useful model for dissecting the complex pathway leading to IL-2 gene expression after TCR engagement (65).

In summary, we have demonstrated directly by a variety of parameters that γ/δ T cells can be subject to positive selection in development. By virtue of the alterations of cell surface phenotype, the functional immaturity, and the absence of peripheral tg-bearing T cells in β_2m^- mice, as well as the sensitivity of γ/δ tg development to CsA, γ/δ lineage selection parallels that of α/β T cells in several fundamental ways. Moreover, examining a class I MHC TL-specific γ/δ tg in a model system analogous to ours, others have obtained very similar results (22). However, it is also true that substantial normal γ/δ T cell development occurs in nontransgenic β_2m^- mice (12). Thus, it will be important to determine if other γ/δ T cells undergo positive selection, especially non-MHC-specific γ/δ T cells, and consequently to establish whether TCR–self-antigen interactions represent a critical step in all T cell differentiation.

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